TOXICITY AND TREATMENT OF RUSSIAN V-AGENT (VR) INTOXICATION IN GUINEA PIGS[1]

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ABSTRACT

VR (O-isobutyl-S-[2-(diethylamino)ethyl]methyl phosphonothioate) is a structural isomer of VX and is thought to be the principal V-agent found in Russian chemical weapons. We evaluated the toxicity (24 hr LD_{50}) of VR and determined the effectiveness of oxime and atropine (ATR) treatment and pyridostigmine (PB) pretreatment in guinea pigs. Lethality dose-response curves for VR were generated in untreated animals and in animals treated with atropine (ATR) and oxime with or without PB pretreatment. PB was injected i.m. 30 min prior to s.c. VR challenge. One min after VR the animals were treated i.m. with ATR (16 mg/kg) plus an equimolar dose of either 2-PAM (25 mg/kg) or HI-6 (55 mg/kg). Mortality was assessed at 24 hours. The LD_{50} of VR was 11.3 ìg/kg. Treatment with ATR plus 2-PAM or HI-6 resulted in protective ratios (PR) of 6.5 and 43.9, respectively. In PB-pretreated animals, the PRs were 7.1 and 28.7, respectively. The results indicate that 1) VR is a highly toxic organophosphorus agent, 2) it can be effectively treated with oximes and atropine, 3) HI-6 is significantly more effective than 2-PAM, and 4) PB pretreatment affords no further enhancement to the efficacy of atropine and oximes. When compared with previous findings in guinea pigs, ATR + 2-PAM is significantly less effective against VR than against VX. This observation may necessitate a careful examination of the best oxime to use as an antidote against V-agents, particularly for personnel involved in international chemical weapons disposal where exposure to VR is possible.

INTRODUCTION

Although both the United States and Russia possess chemical weapons identified as V-agents in their respective chemical munitions stockpiles (Ember,1990; Maynard and Beswick,1992; Federov and Svetlakova, 1995), it has been suggested that the U.S. and Russian V-agents are not identical. Recently this suggestion was confirmed with the identification of O-isobutyl S-[2-(diethylamino)ethyl] methyl - phosphonothioate (VR) as the standard Russian V-agent (Fig 1), a structural isomer of VX, the standard U.S. V-agent (Szafraniec *et al*., 1995).

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"The Research was conducted in compliance with the Animal Welfare Act and other Federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the *Guide for the Care and Use of Laboratory Animals*, National Research Council, 1996. The facility where this research was conducted is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International."

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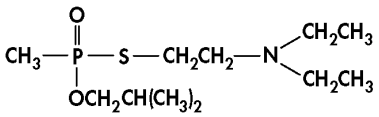

VR

Figure 1. Structure of VR.

The purpose of this investigation was to characterize the toxicity of VR, and determine the effectiveness of pretreatment and treatment countermeasures in an intact animal. Experiments included pretreatment with pyridostigmine bromide (PB) and/or postchallenge treatment with atropine plus an oxime. The oximes used were 2-pyridine aldoxime methyl chloride (2-PAM), the currently fielded U.S. antidote for nerve agent intoxication, and 1-(((4-(aminocarbonyl)pyridino)methoxy)methyl)-2-((hydroxyimino)methyl pyridinium dichloride (HI-6), a bispyridinium oxime that has been widely studied and reported to be more effective than standard oximes (toxogonin and 2-PAM) against nerve agent intoxication, especially soman (Lallement *et al*., 1997). The guinea pig was used as the animal species since it has been generally regarded as the best nonprimate model for assessing the efficacy of pretreatment and treatment countermeasures against organophosphorus (OP) poisoning (Lundy, 1992).

METHODS

Animals: Male Hartley albino guinea pigs (Charles River Laboratories) weighing 300-450 gms were used. Animals were quarantined and observed for a minimum of five days for evidence of disease under an AAALAC accredited animal care and use program prior to being put on study. Guinea pig ration and tap water were provided *ad libitum*. The guinea pig holding rooms were maintained at 21 $\pm$ 2°C with 50% $\pm$ 10% relative humidity using at 12-15 complete air changes per hour of 100% conditioned fresh air. The animals were maintained on a 12-hour light/dark, full-spectrum lighting cycle with no twilight.

Chemicals: VR (O-isobutyl S-[2-(diethylamino)ethyl]methylphosphonothioate) was synthesized by the U.S. Army Edgewood Chemical Biological Center (ECBC), Aberdeen Proving Ground, Maryland. A stock solution was prepared gravimetrically at a concentration of 1 mg/ml in deuterium oxide (D_2O). The VR concentration was verified by gas chromatography with flame ionization detection. Stock solutions were stored in 1 or 5 ml aliquots at -70°C until needed. Dilutions were prepared from thawed stock and maintained on ice. Atropine (ATR) sulfate (Lot# BL09195), 2-PAM (Lot# BK96362), HI-6 (Lot# BM08862), and pyridostigmine bromide (PB) (Lot # BM03894) were obtained through the Walter Reed Army Institute of Research (WRAIR), Washington, D.C. PB, ATR and oximes were prepared in sterile water daily. ATR was admixed with the oxime to provide a single solution for injection.

Agent Toxicity Studies: VR was injected s.c. in the nape of the neck using a dose volume of 1 ml/kg. A lethality dose-response curve was generated in sequential stages using the stage-wise adaptive approach described by Feder *et al*. (1991a, 1991b, 1991c). In each stage 1-2 animals were allocated to each of 4-5 agent challenge levels selected to span the predicted range of lethality from 0-100%. Mortality was assessed 24 hours after exposure. Results of each stage were evaluated and combined to aid in selecting agent doses for the next stage. The stage-wise approach enabled agent dose allocation in later stages to be optimized to better estimate the dose-lethality relations. A total of three experimental stages were conducted. In each stage, animals were randomly assigned to the challenge levels.

Efficacy Studies: The efficacy of the pretreatment and treatment countermeasures was determined using a similar stage approach design described in the previous section. Lethality dose-response curves for VR were generated in saline-treated animals and in animals treated with atropine (ATR) and oxime with or without PB pretreatment. PB (0.026 mg/kg) or saline was injected i.m. 30 min prior to s.c. VR challenge. The dose of PB was selected to give approximately 40% inhibition of erythrocyte AChE 30 min after injection (Lennox *et al*., 1985). One min after VR challenge the animals were treated i.m. with saline or ATR (16 mg/kg) plus an equimolar dose of either 2-PAM (25 mg/kg) or HI-6 (55 mg/kg). Mortality was assessed at 24 hours. In each stage pretreatment, agent doses, and treatments were allocated to the animals randomly.

Data Analysis: Probit models were fitted to the dose-lethality data using the method of maximum likelihood. The models were fitted using the general purpose nonlinear regression procedure, PROC NLIN, in the SAS statistical computing system and specialized programs written by Battelle Memorial Institute (Columbus, Ohio) for the sequential stage approach (Feder *et al*., 1991c). The estimated regression coefficients were used to compute LD_{50} values, protective ratios (PR), predicted survival estimates, and their associated 95% confidence limits. The PR was defined as the ratio of the OP LD_{50} in treated animals divided by the OP LD_{50} in untreated animals.

RESULTS

VR Toxicity: Table 1 summarizes the toxicity of VR in guinea pigs. The 24-hour LD_{50} was 11.3 ug/kg with a 95% confidence interval of 10.5 to 12.1 ug/kg. The slope of the dose-lethality curve was 19.5. The sample size of 56 was obtained by pooling the data from the acute toxicity study and the saline control group in the efficacy study. The two studies were not different statistically.

TABLE 1. Lethality of VR in Guinea Pigs.

N[1]	Slope (ìg/kg, s.c.)	24hr LD_{50}	95% C.I.
56	19.5	11.3	10.5 - 12.1

1. Combined data from 2 studies- see Results

Countermeasure efficacy: Tables 2, 3, and 4 summarize the effectiveness of atropine and oxime treatment with or without PB pretreatment against lethal intoxication by VR. Table 2 presents the data as protective ratios (PR), while Table 3 and 4 present the data as predicted survival against varying exposure levels of the OPs.

The PR for 2-PAM + atropine treatment of VR intoxication without PB pretreatment was 6.5, while the PR for HI-6 + atropine was 43.9. The PR for HI-6 was significantly greater ($p<0.05$) than the PR for 2-PAM (Table 2).

TABLE 2. Effectiveness of Medical Countermeasures Against VR Intoxication in Guinea Pigs.

Treatment	Protective Ratio (95% C.I.) - PB	+PB
Atr + 2-PAM	6.5 (5.4-7.7)	7.1 (5.8-8.6)
Atr + HI-6	43.9* (35.2-54.8)	28.7 (18.5-44.5)

* $p<0.05$ compared with Atr + 2-PAM

The disparity in the efficacy between 2-PAM and HI-6 against VR can be further seen from the predicted survival data in Table 3. Survival in 2-PAM-treated animals was 100% at 2 LD_{50}s, 81% at 5 LD_{50}s but only 7% at 10 LD_{50}s, while in the HI-6-treated guinea pigs 100% survival was maintained over the entire range of VR doses from 2-10 LD_{50}s.

TABLE 3. Predicted Survival Against VR Intoxication Following Treatment with Atropine and Oximes.

No. VR LD_{50}s	(LCL - *% Survival* - UCL)[1] Atr + 2-PAM	Atr + HI-6
2	100 - *100* - 100	100 - *100* - 100
3	96 - *100* - 100	100 - *100* - 100
4	79 - *95* - 100	100 - *100* - 100
5	55 - *81* - 100	100 - *100* - 100
6	36 - *61* - 85	100 - *100* - 100
7	18 - *40* - 62	100 - *100* - 100
8	0 - *24* - 48	100 - *100* - 100
9	0 - *14* - 35	100 - *100* - 100
10	0 - *7* - 25	100 - *100* - 100

1. LCL = Lower 95% Confidence Level; UCL= Upper 95% Confidence Level

PB pretreatment did not enhance the efficacy of ATR and oxime therapy against VR intoxication. The PR in PB-pretreated animals treated with ATR+ 2-PAM was 7.1 compared with 6.5 in nonpretreated animals, and the PR in PB-pretreated animals treated with HI-6 + ATR was 28 compared with 43.9 in animals not pretreated; this difference was not significant (Table 2). The predicted survival data in Table 4 demonstrates further that PB provided no enhancement of Atr + oxime efficacy. There was little change in expected survival over a VR exposure range of 2 to 10 LD_{50}s in PB (Table 4) vs non-PB-pretreated (Table 3) animals. In the HI-6-treated animals, there was a trend for survival in the PB-pretreated animals (Table 4) to be reduced over the challenge range compared with nonpretreated animals if the worse case scenario (i.e., survival at the lower 95% CI) is considered (Table 4).

TABLE 4. Predicted Survival Against VR Intoxication Following Pretreatment with PB and Treatment with Atropine and Oximes.

No. of VR LD_{50}s	(LCL - *% Survival* - UCL)[1] Atr + 2-PAM	Atr + HI-6
2	100 -*100*- 100	100 - *100* - 100
3	98 -*100*- 100	98 - *100* - 100
4	86 - *97*- 100	95 - *100* - 100
5	63 - *87* - 100	91 - *99* - 100
6	42 - 70 - 99	87 - *97* - 100
7	25 - *51* - 77	82 - *96* - 100
8	10 - *34* - 57	78 - *95* - 100
9	0 - *21* - 43	73 - 93 - 100
10	0 - 13 - 31	68 - *91* - 100

1. LCL = Lower 95% Confidence Level; UCL= Upper 95% Confidence Level

DISCUSSION

Our studies indicate that VR was a highly toxic organophosphorus agent in the guinea pig with a LD_{50} similar to VX (9.0 ug/kg.s.c.) (Koplovitz *et al*., 1992). VR was also more toxic than soman, sarin, cyclosarin (GF) and tabun (Koplovitz, 2000). The slope of the VR dose-lethality curve was very steep indicative of a very specific mechanism of toxicity. In this regard, Maxwell et al. (1997) found that VR was a potent inhibitor of AChE with a bimolecular rate constant 3-fold greater than the inhibition rate constant for VX.

Our results indicate that VR was very treatable with atropine and oximes, with HI-6 being 7-fold more effective than 2-PAM. The greater efficacy of HI-6 over 2-PAM was likely due to differences in reactivating ability. This is supported by studies showing that HI-6 was a 5-fold better (faster) reactivator of VR-inhibited AChE than was 2-PAM *in vitro* (Maxwell *et al.*, 1997). The PR of 6.5 that we found with atropine +2-PAM treatment is significantly less than what this treatment regime has been reported to achieve against VX. Maxwell *et al*. (1997) reported a PR against VX of 36.9 using the same doses of atropine and 2-PAM and the same treatment time as used in the present study. Inns and Leadbeater (1983) reported a PR of 25 using an atropine dose of 17.4 mg/kg and a P2S dose of 30 mg/kg. In the latter study, 1/2 of the P2S dose was given 10 min prior to VX challenge, and the rest was administered 1 min after challenge together with the atropine. Koplovitz *et al*. (1992) reported a PR of 59 using atropine 32 mg/kg and 2-PAM 25 mg/kg administered 1 min after challenge. The greater efficacy of 2-PAM against VX compared with VR, however, cannot be explained as convincingly based on differences in enzyme reactivation. Maxwell *et al*. (1997) reported that 2-PAM was only 1.6-fold better in reactivating VX-inhibited AChE than VR-inhibited enzyme *in vitro*. However, *in vivo* 2-PAM appears to be approximately 6-fold more effective against VX than it is against VR . It may be that other factors such as differences in the distribution between the VX and VR could account for the difference in the efficacy of 2-PAM (Maxwell *et al.*, 1997). Ligtenstein *et al*. (1991) have observed that oximes are more effective against OP compounds whose distribution is predominately peripheral in comparison to OP compounds that distribute in the central nervous system. Maxwell *et al*. (1997) also found that VR has a lower pK_a than VX, which may allow more VR to distribute into the central nervous system, making it less susceptible to oxime treatment.

The failure of PB pretreatment to enhance the efficacy of atropine and oxime treatment of VR intoxication was similar to previously reported results against VX and sarin (Koplovitz *et al*.,1992). In the latter study, the authors hypothesized that the inability of PB to enhance the efficacy of atropine and 2-PAM treatment of VX and sarin intoxication might be due to several factors. First, these OPs are easily reactivated by 2-PAM, and second 2-PAM is a more effective reactivator of phosphonylated than of carbamylated AChE . Therefore, when the rate of oxime-induced reactivation is absolutely critical to the demonstration of efficacy (*e.g.*, when lethal doses of OPs are used), there would be more phosphonylated enzyme available for reactivation in the absence of PB pretreatment than in the presence of PB. The results of the present study lend further support to the contention that OPs, which are easily reactivated with oximes show no benefit from PB pretreatment. Also, it would appear that the more effective the oxime is in reactivating the phosphonylated enzyme the greater the likelihood of a negative interaction in the presence of PB. In our studies, PB had a more dramatic effect on the efficacy of atropine and HI-6 than on the efficacy of atropine and 2-PAM.

The PR, historically, has been the standard method of representing the efficacy of medical countermeasures against nerve agent intoxication; however, PRs can be misleading if not completely understood. The tendency (for those who are not experts in the field) is to interpret a PR as the challenge level against which complete protection (100%) can be provided, while in fact, by definition the survival at any given PR is only 50%, since it is the ratio of agent LD_{50} doses (treated/untreated). Also, the PR does not give any information on survival (mortality) at higher or lower challenge levels, nor does it provide information on the slope of the dose-response. Predicted survival, on the other hand, provides information on survival (mortality) over a range of challenge doses of interest and also gives an indication of the slope of the response. We feel it is a more practical and useful method of presenting this type of data. For example, although the PR for atropine and 2-PAM is 6.5 (Table 2), the data in Table 3 show that survival is reduced to 81% at 5 LD_{50}s and could be as low as 55% based on the lower limit of the 95% confidence interval. Similarly, at 4 LD_{50}s, survival could be as low as 79%. The PR does not give the same amount of information as predicted survival over a range of exposure levels.

CONCLUSIONS

1. VR is as toxic as VX.
2. VR is significantly less treatable with atropine and 2-PAM.
3. HI-6 is significantly more effective than 2-PAM in treating VR intoxication.
4. PB pretreatment does not enhance the efficacy of atropine and oxime treatment of VR intoxication.
5. Depending on the level of exposure, the choice of oxime for treatment of VR intoxication needs to be carefully considered.

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